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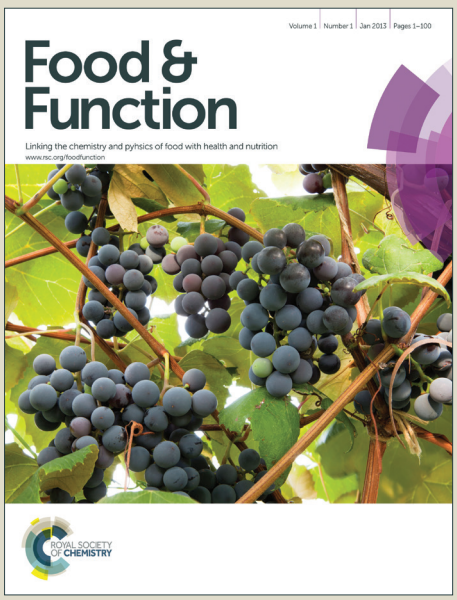

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ARTICLE

Peptides present in the non-digestible fraction of common beans (*Phaseolus vulgaris* L.) inhibit angiotensin-I converting enzyme by interacting with its catalytic cavity independently of their antioxidant capacity

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The aim was to evaluate the angiotensin-I converting enzyme (ACE) inhibitory potential and the antioxidant capacity of pure synthesized peptides (GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY) originally identified in the non-digestible fraction (NDF) of common bean (P. vulgaris L.) that had previously demonstrated antiproliferative activity against human colorectal cancer cells. The five peptides were able to inhibit ACE with half maximal inhibitory concentration (IC_{50}) values ranging from 65.4 (GLTSK) to 191.5 (MPACGSS) µM. The combination of GLTSK and MTEEY increased ACE inhibition by 30% compared to equieffective doses of the single peptides. According to molecular docking analysis, the five peptides had lower estimated free energy values (-6.47 to -9.34 kcal/mol) when they interacted with the catalytic site of ACE than with the substrate hippuryl-histidyl-leucine (-5.41 kcal/mol), thus inhibiting the enzymatic activity. According to the molecular docking analysis, the five peptides interacted with four (His353, Ala354, Glu411 and Tyr523) out of 6 catalytic residues. Moreover, MPACGSS had the highest antioxidant activity according to FRAP (421.58 µmol $FeSO_4$/mg), Fe2+ chelation (2.01 µmol Na2EDTA/mg) assays, and also in DPPH (748.39 µmol Trolox/mg of dry peptide) and ABTS (561.42 µmol Trolox/mg) radicals scavenging assays. The results support the hypothesis that peptides present in the non-digestible fraction of common bean (Phaseolus vulgaris L.) may exert their physiological benefits independently of their antioxidant capacity, by ACE inhibition through interaction with its catalytic cavity.

1. Introduction

Common bean (Phaseolus vulgaris L.) is a legume consumed worldwide. It is considered a nutraceutical food due to its high content of bioactive compounds and macromolecules, namely resistant starch, oligosaccharides, polyphenols, and protein1. The consumption of common bean has been related to a decrease in the risk to develop non-communicable diseases such as diabetes, cardiovascular diseases, and colorectal cancer[1,2,3]. The latter is the third most common cancer in men and the second in women around the world[4]. The potential anti-colorectal cancer effect of common bean has been evaluated and attributed to the combined activity of the bioactive compounds present in its non-digestible fraction, such as polyphenols and carbohydrate fermentation products[1]. However, the bean non-digestible fraction (NDF) also contains a significant amount (around 17%) of protein and/or peptides that may have potential benefits[5]. The non-digestible fraction is a portion of the food that resists gastrointestinal digestion until reaching the colon carrying several bioactive compounds[6]. Recently we characterized the protein portion of common bean NDF and found that five peptides comprised roughly 70% of the total protein detected: GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY. Furthermore, these peptides were able to inhibit in vitro proliferation of HCT116 and RKO human colorectal cancer cells in a dose-response manner. In addition, they were able to modulate proteins and genes related to cell cycle arrest and apoptosis induction in ways that would lead to a reduction of cancer growth[7,8]. According to an in silico study using the bioinformatics tool Biopep, the aforementioned peptides have potential to inhibit angiotensin-I converting enzyme (ACE)[7], an important member of the Renin-Angiotensin-Aldosterone system recently considered as a

possible target for colorectal cancer treatment due to its relation with angiogenesis and cell proliferation[9]. Several studies have shown the beneficial properties of common bean protein hydrolysates, highlighting ACE inhibition and antioxidant activities[10], which also can contribute to the chemoprevention of colorectal cancer. However, no studies have proven the efficacy of pure peptides, originally present in common bean NDF, to inhibit ACE.
On the other hand, free radicals are generated in homeostatic conditions in the body during respiration in aerobic organisms, and can exert preventive roles against infection. However, the imbalance in free radicals results in cellular damage, which can trigger mechanisms related to diseases including atherosclerosis, diabetes and cancer[11]. In addition to the physiological production of pro-oxidants and their secondary reactions, there are other sources such as the products of oxidation of food constituents, or excessive amounts of metals in the body[12,13]. Therefore, proteins, antioxidant peptides and amino acids from dietary sources can also contribute to defending the body against oxidation by inhibiting free radicals and chelating transition metals. The aim of this investigation was to evaluate ACE inhibitory potential and the antioxidant capacity of pure peptides (GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY) originally identified in the NDF of common bean. Additionally, the isobologram analyses of ACE inhibitory activity due to the interaction of peptides, and in silico analysis of its inhibition were performed.

2. Materials and methods

2.1 Materials

Peptides GLTSK, LSGNK, GEGSGA, MPACGSS and MTEEY were synthesized by GenScript (Piscataway, NJ, USA) with a purity > 98% based on the composition found in the bean NDF. Chromatograms and mass spectra analysis are shown in Supplementary Figure 1. Acetonitrile HPLC grade was purchased from J.T. Baker® (Center Valley, PA, USA). Trifluoroacetic acid HPLC grade was purchased from Karal (Guanajuato, México). ACE from rabbit lung (cat. num. A6778), pepsin from porcine gastric mucosa (cat. num. P7000), pancreatin of porcine pancreas (mixture of trypsin, amylase and lipase, ribonuclease, and protease, produced by the exocrine cells of the porcine pancreas, cat. num. P7545) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

2.2 Measurement of ACE-inhibitory activity

The ACE inhibitory activity was measured by the method of Wu et al.[14], with slight modifications. Briefly, a total reaction volume of 90 µL was made up of 50 µL of 2.17 mM hippuryl-histidyl-leucine (HHL), 30 µL of 2 mU of ACE and 10 µL of different concentrations of captopril as an inhibition control or peptides, all prepared with 100 mM borate buffer, containing 300 mM NaCl, pH 8.3. The HHL and the captopril or peptides were maintained at 37 °C for 15 min. ACE solution was also maintained at 37 °C for 10 min before the two solutions were combined and incubated at 37 °C with continuous agitation. A blank sample was prepared by replacing the inhibitor solution with the 100 mM borate buffer. The reaction was terminated after 60 min of agitation by addition of 70 µL of 1 M HCl and the solution was filtered through a 0.45 µm x 13 mm nylon syringe filter. A high-performance liquid chromatography-diode array detection (HPLC-DAD) analysis was conducted in an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) using a Poroshell 120 SB-C18 column (4.6 mm x 150 mm, 2.7 µm, Agilent Technologies). The column was thermostatically controlled at 30 °C ± 0.6 and the flow rate was set to 0.5 mL/min. The mobile phase consisted of two solvents. Solvent A was water adjusted with 0.05% trifluoroacetic acid and solvent B was acetonitrile 0.05% trifluoroacetic acid. A linear gradient was used as follows: 5 - 60% solvent B for the first 10 min, maintained for 2 min at 60% B, then returned to 5% B for 1 min. Detection of hippuric acid (HA) and HHL was performed at 228 nm. A volume of 10 µL was injected and the samples were analyzed in triplicate. The percentage of ACE inhibition was calculated according to the following equation:

$$ACE\ inhibition\ (\%) = \left[1 - \left(\frac{A_{inhibitor}}{A_{blank}}\right)\right] x\ 100$$

Where $A_{inhibitor}$ and A_{blank} are peak areas corresponding to HA for an inhibitor sample and for the blank, respectively. The IC50 values were defined as the concentration of inhibitor required to reduce the HA peak area by 50% compared to the blank.

2.3 Isobologram analysis of ACE inhibitory activity by bean peptides

Since the peptides analyzed in this study were originally identified in a bean extract where they were initially present in combination, we selected the three most potent peptides (GLTSK, MTEEY and GEGSGA) inhibiting ACE activity to be evaluated by pairs for potential interactions. The interactions were validated by isobolographic analysis in which the combinations were comprised of equieffective doses of the individual components[15]. Using the IC_{50} values of each peptide, the additive line was plotted and the equieffective dose was calculated. Subsequently, a dose–response curve of the ACE inhibition was obtained in a fixed-ratio for the mixture of peptides (1:1) that was based on the IC_{50} values

of each individual peptide. The experimental IC_{50} values for the peptide combinations were calculated. In an isobologram, when the peptide combination IC_{50} lies on the theoretical IC_{50} add line, then the mixture is considered to be additive. If the IC_{50} of the combination lies below the theoretical IC_{50} add line, the mixture is considered to be synergistic. An interaction index (γ) was calculated according to the formula: IC_{50} combination / IC_{50} theoretical. Gamma values near 1 indicated additive interaction; > 1 implied an antagonistic interaction and < 1 indicated a synergistic interaction[16].

2.4 ACE inhibition and peptide structure stability after gastrointestinal simulated digestion

To evaluate structural stability, a mixture solution of HPLC-grade water containing 0.1 mM of each peptide was prepared and analyzed with HPLC-DAD using a Poroshell 120 SB-C_{18} column (4.6 mm x 150 mm, 2.7 µm, Agilent Technologies). The column was thermostatically controlled at 30 °C ± 0.6 and the flow rate was set to 0.5 ml/min. The mobile phase consisted of two solvents. Solvent A was water adjusted with 0.05% trifluoroacetic acid and solvent B was acetonitrile 0.05% trifluoroacetic acid. A linear gradient was used as follows: 5-70% solvent B for the first 14 min and then returned to 5% B for 1 min. The peptides were detected at 228 nm. The peak area of each peptide was recorded. The solution was kept at 37 °C for 10 min and adjusted to pH 2. Pepsin was added at a concentration of 5% w/v and allowed to react at 37 °C for 1.5 h. Then, pH was adjusted to 7.5 and pancreatin was added at a concentration of 5% w/v and allowed to react at 37 °C for 1.5 h. Reactions were terminated by incubating for 15 min at 75 °C. After centrifugation at 12,000 x g for 15 min, the supernatant was transferred, adjusted to pH 8.3 and filtered through a 0.45 µm x 13 mm nylon syringe filter in order to be analyzed with HPLC-DAD with the above conditions. The peak areas and retention times of the pre- and post- incubated peptide solutions were compared for potential differences caused by the digestive enzymes incubation.

To study the stability of the peptides to inhibit ACE, pure peptides were dissolved at a molarity equivalent to 50 µg/mL in HPLC-grade water and kept at 37 °C for 10 min, pH was adjusted to 2. The peptides were incubated with pepsin/pancreatin using the same conditions as above. After incubation, the peptides were assayed for ACE-inhibitory activity as previously described. The ratio of ACE-inhibition percentage per concentration (µM) of pre- and post- incubated peptide solutions was compared to the observed potential differences caused by incubation with digestive enzymes pepsin and pancreatin.

2.5 In silico analysis of ACE inhibition

In order to explore the structural mechanism by which these peptides present in common bean NDF inhibit ACE, an *in silico* analysis through molecular docking was performed. Docking calculations were carried out using DockingServer[17]. Peptides were designed using Instant MarvinSketch (ChemAxon Ltd.) The MMFF94 force field[18] was used for energy minimization of ligand molecules, peptides and captopril (Supplementary Figure 2) using DockingServer. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on human testicular angiotensin I-converting enzyme (1UZE) protein models. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools[19]. Affinity maps of 20×20×20 Å grid points and 0.375 Å spacing were generated using the Autogrid program[19]. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method[20]. Initial position, orientation, and torsions of the ligand molecules were set randomly. Each docking experiment was derived from 100 different runs that were set to terminate after a maximum of 2,500,000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

2.6 Antioxidant capacity assays

Ferric reducing antioxidant power (FRAP)

FRAP values were obtained according to the method reported by Firuzi *et al.*[21]. Briefly, 25 µL of peptides were dissolved (1 mg/mL) and placed in a 96-well microplate. Then, 175 µL of freshly prepared and warm (37 °C) FRAP solution was added. The absorbance at 595 nm was monitored at 0, 4, 10, 30, 60, and 90 min. Blanks were prepared and a standard curve of $FeSO_4$ was obtained ($y=0.0015x-0.0557$, $R^2=0.98$). The results were expressed as µmol of $FeSO_4$ equivalents per milligram of peptide (µmol $FeSO_4$/mg) at 90 min.

DPPH method

The estimation of the Trolox equivalent antioxidant capacity (TEAC) was determined using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to the method reported by Nenadis et al.[22] A total of 20 µL of peptide solution in water (2 mg/mL) was mixed with 200 µL of 150 µM of DPPH in 80% methanol. The measurement was performed in triplicate. The absorbance was read at 520 nm after 0, 4, 10, 30, 60, and 90 min. The TEAC value was calculated using Trolox as standard for the calibration curve ($y=-0.0012x+0.9822$, $R^2=0.99$), and expressed as µmol of Trolox equivalents per milligram of peptide (µmol Trolox/mg).

ABTS method

TEAC estimation was performed using the 2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay described by Loarca-Pina et al.[23] A 20 µL of peptide solution (2 mg/mL) was mixed with 230 µL of $ABTS^{\cdot+}$ radical solution. The absorbance was read at 570 nm at room temperature. The measurement was performed in triplicate. The TEAC value was calculated employing a Trolox calibration curve ($y=-0.0016x+0.975$, $R^2=0.99$) and expressed as µmol of Trolox equivalents per milligram of peptide (µmol Trolox/mg).

Metal chelating activity

Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex[24]. Dissolved peptides at a concentration of 1 mg/mL (30 µL) were mixed with 150 µl of 2 mM $FeCl_2$. Ferrozine (5 mM) was added after incubation for 30 min at room temperature. Na_2EDTA was used as a positive control to build a standard curve ($y=-0.1536x+0.7639$, $R^2=0.99$). Binding of Fe(II) ions to ferrozine generated a colored complex that was measured at 562 nm, using a microplate reader. The results were expressed as µmol Na_2EDTA/mg peptide.

2.6 Statistical analysis

The results were expressed as the mean ± standard deviation of three independent experiments run in at least a duplicate and analyzed through ANOVA. Colorimetric assays were performed in three independent replicates with at least two repetitions in each plate. Statistical significance was determined using Student's t-test (α=0.05) for comparing mean pairs and Tukey test (α=0.05) for multiple mean comparison using software JMP version 7.0. Correlation analysis of the properties evaluated in this study was performed using GraphPad® Prism version 6.05.

3. Results

3.1 Peptides with ACE-inhibitory activity, their interaction, and stability after gastric enzymes-digestion

The peak area of the product of ACE reaction (HHL) decreased as the peptide concentration increased (Figure 1A), indicating that peptides inhibited ACE as a function of concentration (Figure 1B). The ACE inhibitory activity was also expressed as IC_{50} values, which indicated the concentration (µM) of each peptide required to inhibit by 50% the enzymatic activity of ACE. The lowest IC_{50} value was for GLTSK (65.4 µM) and the highest was for MPACGSS (191.5 µM) (Figure 1C). The results were significantly higher than the obtained for captopril (17.5 µM); however, the five peptides tested had higher (less potent) ACE-inhibitory activity than previous reports of common bean protein hydrolysates[25].

The resulting isobolograms of the peptides interaction inhibiting ACE activity are presented in Figure 2. It was observed that two combinations were additive (MTEEY-GEGSGA, and GLTSK-GEGSGA); only the combination of GLTSK and MTEEY peptides had synergistic interactions reducing by approximately 30% the concentration needed to inhibit 50% of the enzymatic activity.

In the present study, the peptides were subjected to an *in vitro* subsequential digestion using pepsin and pancreatin. The percent of ACE inhibition, using concentrations of molarity equivalent to 50 µg/mL of each peptide subjected to digestion, is summarized in Figure 3A. The digestive enzymes did not show impact on the peptides LSGNK, GEGSGA and MPACGSS to inhibit ACE, and only a modest significant ($p < 0.05$) reduction of ACE inhibitory activity by GLTSK and MTEEY peptides was observed. These results were confirmed evaluating the elution time of the different peptides, finding no significant differences (Supplementary Figure 3). In Figure 3B it can be observed that the incubation with pepsin and pancreatin had no impact in the peak area of the peptides.

3.2 Molecular docking study of peptides inhibiting ACE

The minimum estimated free energy of the interactions of peptides with ACE is shown in Table 1. Estimated free energy indicates that compounds with a more negative value are more likely to inhibit the enzyme. The peptides studied had free energy values ranging from -6.47 to -9.34 kcal/mol, while substrate HHL had a free energy value of -5.41 kcal/mol. Captopril was also evaluated as a control, presenting a value of free energy of -4.95 kcal/mol. All the atom distances were shorter than 4 Å. The most stabilized pose of the peptide bonds with ACE were obtained, an example can be observed in Figure 4A and 4B. All peptides studied were able to interact with several amino acid residues of ACE catalytic cavity, ranging from 11 to 26 interactions depending on the peptide (Table 1). These peptides were able to inhibit ACE activity mainly through contribution of hydrogen bonds, hydrophobic, polar and cation π (Figure 4C).

3.3 Antioxidant and metal chelating activities

The peptides studied in this investigation exerted antioxidant activity (Table 2). General trends were observed within the results using different methods as determined by the Tukey test (p<0.05). For instance, MPACGSS had the highest values of antioxidant capacity followed by MTEEY and LSGNK, and finally the lowest values were presented by GLTSK and GEGSGA. Regarding FRAP assay, the values ranged from 73.8 to 421.58 µmol $FeSO_4$/mg. Also, Table 2 shows the ability of the peptides to scavenge $DPPH^{\cdot+}$ and $ABTS^{\cdot}$ radicals. The values obtained for ABTS scavenging activity ranged from 18.14 to 561.42 µmol Trolox/mg, and for DPPH scavenging activity from 49.98 to 748.39 µmol

Trolox/mg. Moreover, the chelating activity of peptides expressed as μmol Na_2EDTA/mg ranged from 1.01 to 2.01.

4. Discussion

One of the smallest peptides inhibited ACE activity more efficiently (GLTSK, Glu-Leu-Thr-Ser-Lys), agreeing with several reports which showed that shorter peptides have higher probability to access the catalytic cavity of ACE[26]. Since these peptides were originally found in an extract of the NDF of common bean cultivars Bayo Madero, Azufrado Higuera and Negro 8025 and had antiproliferative effect against human colorectal cancer cells, it is important to explore potential inhibitory mechanisms[8]. ACE is a member of the Renin-Angiotenin-Aldosterone system (RAAS) that primarily functions to regulate important cardiovascular processes[27]. However, in several studies, protein overexpression and the increased activity of RAAS in neoplastic colonic tissue have been observed. Furthermore, it has been established that an increase of ACE activity is related to proliferation, angiogenesis, and metastasis of colon cancer[28]. The intake of ACE inhibitory drugs has shown to significantly decrease the re-incidence of colon adenomatous polyps in diagnosed patients[29]; also, ACE inhibitors have proven to slow or stop the metastatic process[30,31]. However, the use of pharmacological ACE inhibitors can trigger undesirable side effects including allergic reaction, skin conditions, bone marrow suppression, nephrotic syndrome among others[32]. Due to these adverse effects, a possible coadjuvant strategy is the intake of natural dietary sources of ACE inhibitors, such as the peptides described in this study. In addition to inhibitory activity, peptides have to survive possible hydrolysis, either gastrointestinal proteases of the digestive tract or the remaining proteolytic activity of ileal and pancreatic effluents reaching the colon. Since the structure and concentration of a compound is directly related to the retention time and peak area, the maintenance of those parameters after incubation with pepsin and pancreatin suggests that the peptides used in this study showed resistance to simulated digestion.

Although only one combination of peptides showed synergistic ACE inhibition, the presence of several peptides in the non-digestible fraction of common bean represents an advantage to inhibiting the enzyme. A synergistic effect of peptides inhibiting ACE has been observed on dipeptides extracted from hemp seed (*Cannabis sativa* L.) protein, suggesting that the peptides could bind to other sites than the catalytic cavity of the enzyme contributing to ACE inhibition[33].

Although molecular docking showed that the free energy of the positive control captopril was higher compared to the peptides studied, the IC_{50} value was significantly lower. This can be explained by the stereochemical configuration that appears to play a significant role in the inhibitory mechanisms, as smaller molecules have higher probabilities to access the catalytic site of ACE[26]. Furthermore, the peptides had lower values of free energy compared to the substrate HHL, suggesting that they have more affinity for the catalytic site, thus exerting their ACE inhibitory activity. As previously reported, since the active sites of ACE are protected by an N-terminal lid that blocks the access of large polypeptides, the smallest peptides had the lowest IC_{50} and free energy interaction values[26]. ACE has six reported catalytic residues inside its cavity, from which common bean NDF peptides were able to interact with His353, Ala354, Glu411 and Tyr523. The predicted free energy of the peptides within the catalytic cavity correlated with IC_{50} values ($r = 0.77$), confirming that the two approaches used in ACE inhibition complemented each other.

Beyond ACE inhibition, the antioxidant activity is considered also as a chemoprotective mechanism of action against colorectal cancer[34] therefore, it is a desirable property in dietary bioactive compounds. We evaluated the antioxidant activity of the peptides using different methods based on the fact that each method can help to elucidate the different mechanisms by which peptides contribute to free radical stabilization.

The FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-s-triazine complex [$Fe3^+$- (TPTZ)2] 3^+ to the intensely blue colored ferrous complex [$Fe2^+$-(TPTZ)2] 2^+ in acidic medium[35]. The reducing capacity of the peptides may serve as a significant indicator of its potential antioxidant activity as an electron-donating reducing agent which can donate an electron to a free radical. As a result, the radical is neutralized, and the reduced species subsequently acquire a proton from the compound[36]. Previous reports indicate that hydrophobic and sulfur amino acids of peptides contribute to the reducing power[37]. This is in agreement with our results since those types of amino acids are present in the peptide MPACGSS, which was the most potent peptide to reduce [$Fe3^+$- (TPTZ)2] 3^+. Another antioxidant mechanism is scavenging of free-radical. In this study, we used the assays based upon $DPPH^{\cdot+}$ and $ABTS^{\cdot}$ radicals. Peptides are believed to intercept the free-radical chain of oxidation and donate hydrogen from the phenolic, imidazole and indole groups present in some amino acids, thereby forming stable end-products that do not initiate or propagate further oxidation[38]. Our results were lower than previous reports[39] for common bean protein hydrolysates with antioxidant activity; this could be explained by the presence of diverse peptides in the whole hydrolysate, conferring higher antioxidant capacity to the samples.

Additionally, transition metal ions, such as Fe^{2+} are able to promote the generation of reactive oxygen species. Fe^{2+} can also catalyze the Haber-Weiss reaction and induce superoxide anions to form more hazardous hydroxyl radicals, which can react with neighboring molecules to

cause severe tissue damage[40]. Therefore, the chelation of transition metal ions by antioxidative peptides could slow the oxidation reaction. As in FRAP and free radical scavenging assays, MPACGSS had the highest ($p < 0.05$) antioxidant activity, but in this case, it was statistically similar to both GLTSK and GEGSGA. The peptides MTEEY and GLTSK had the lowest values with no significant differences between them. Carboxyl and amino group in the side chains of the acidic and basic amino acids present in these peptides are thought to play an important role in chelating metal ions[41], in turn providing their metal chelating activity.

The peptides evaluated in this study presented potential biological properties; interestingly, ACE inhibition did not correlate with the antioxidant and metal chelating capacities. These findings suggest that the peptides could exert their potential benefits by complementarity processes. Certain peptides present in common bean NDF could participate in inhibiting the enzymatic activity of ACE, while other peptides would contribute to enhancing an antioxidant microenvironment. Therefore, the peptides originally found in the NDF of common beans, taken as a whole, present potential to ameliorate oxidative stress and increased ACE activity, both processes related to the reduction of non-communicable diseases, including colorectal cancer.

Conclusion

To the best of our knowledge, this is the first report of the potential bioactive effects in parallel, ACE inhibition, antioxidant and metal-chelating capacities, of pure peptides similar to those present in the NDF of common beans (*P. vulgaris* L). This investigation demonstrated that the peptides can inhibit ACE, with GLTSK being the most effective peptide in both *in vitro* and *in silico* approaches. Also importantly, we have demonstrated that peptides can interact synergistically to enhance their ACE inhibitory potential. The peptides studied presented antioxidant and metal-chelating capacity independently of their ACE inhibition capacity, with MPACGSS the most potent peptide to stabilize free radicals and chelating Fe^{2+}. In summary, our results support a potential bioactive role of bean peptides protecting cells from oxidative stress and, in transformed cells, acting via targets related to ACE-dependent cell proliferation signaling pathway. Further research regarding mechanistic studies and antiproliferative effect of pure peptides in human colorectal cancer cells will determine the impact of the peptides in ACE pathway and their contribution to chemoprotection.

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Table 1 Estimated free energy binding and chemical interactions among peptides present in common bean (*Phaseolus vulgaris* L.) and Angiotensin-1 converting enzyme (ACE) catalytic site.

Compound	EFE1 (kcal/mol)	Residues which had interaction with the ligands and their atom distances (Å)			
		Hydrogen bonds	Polar	Hydrophobic	Cation-π
GLTSK	-9.34	GLU162(2.59), HIS353(2.77), GLU403(2.92), GLU411(3.47), TYR523(3.07)	GLU162(2.80), HIS353(3.51), TYR360(3.16), HIS383(3.84), GLU384(2.60), TYR394(3.89), GLU403(3.05), HIS410(3.15), TYR523(3.14)	HIS353(3.27), ALA354(3.82), HIS383(3.65), PHE391(3.06), PRO407(3.72), HIS410(3.86), PHE512(3.78), HIS513(3.85)	HIS353(3.82), TYR360(3.85), HIS383(3.28), PHE391(3.69)
LSGNK	-9.25	GLU143(2.46), SER355(3.30), PRO407(2.68), GLU411(2.66), TYR523(2.62)	ASN70(2.87), GLU143(2.13), SER355(3.89), HIS387(3.11), GLU411(1.68), SER516(3.62), ARG522(3.22), TYR523(3.74)	HIS353(3.61), PHE391(3.45), HIS410(3.37), PHE512(3.39), VAL518(3.84)	HIS387(3.12), HIS410(3.71), PHE512(3.61)
MTEEY	-7.43	ALA356(3.21)	ASN66(3.24), ASN70(3.73), SER355(3.59), GLU403(3.81), GLU411(2.98), ARG522(2.99)	HIS387(3.55), PHE391(3.25), PRO519(3.53)	TRP357(3.15), HIS387(3.72)
MPACGSS	-7.48	-	SER355(3.32), TYR394(3.87), GLU403(3.16), HIS410(3.52), GLU411(2.98), ARG522(3.47)	HIS387(3.54), PHE391(3.44), HIS410(3.52), VAL518(3.44)	PHE512(3.62)
GEGSGA	-6.47	GLU403(2.47)	GLU143(3.85), TYR360(3.69), TYR394(3.08), GLU403(2.57), HIS410(3.86), ARG522(3.53), TYR523(3.63)	PHE391(3.40), VAL518(3.51)	HIS353(3.84), PHE512(3.27)
HHL2	-5.41	ASN70(2.46), SER355(3.54), SER516(1.98)	GLU143(3.24), SER355(2.57), TRP357(3.14), HIS387(2.89), GLU411(3.65), ARG522(3.18)	LEU139(3.57), TRP357(2.95), HIS387(3.78), VAL518(3.64)	-
Captopril	-4.95	-	HIS353(3.24), GLU384(3.15), HIS513(3.42), TYR523(3.61)	HIS383(3.31), HIS513(3.60), TYR523(3.70)	HIS383(3.52)

[1]EFE: Estimated free energy, defined as the predicted Gibbs free energy between the ligand and the catalytic site of ACE; negative values means spontaneous reactions.

[2]HHL: Hippuryl-histidyl-leucine, substrate of the reaction

Table 2 Antioxidant and metal chelating activities of most abundant peptides present in common bean (*Phaseolus vulgaris* L) non-digestible fraction.

Peptide	FRAP[1]	TEAC (ABTS)[2]	TEAC (DPPH)[2]	Chelating activity[3]
MPACGSS	421.58 ± 30.67^{a}	561.42 ± 2.35^{a}	748.39 ± 0.81^{a}	2.01 ± 0.011^{a}
MTEEY	311.80 ± 18.66^{b}	302.73 ± 10.60^{b}	662.13 ± 0.28^{b}	1.01± 0.089^{c}
LSGNK	139.58 ± 6.33^{c}	40.49 ± 7.87^{c}	55.39 ± 8.33^{c}	1.18 ± 0.003^{bc}
GEGSGA	80.91 ± 6.33^{d}	18.14 ± 2.17^{d}	52.88 ± 3.17^{c}	1.83 ± 0.025^{ab}
GLTSK	73.80 ± 7.68^{d}	28.32 ± 4.86^{cd}	49.98 ± 4.35^{c}	1.80 ± 0.004^{ab}

The results are expressed as the mean ± standard deviation of three independent experiments

[1] Expressed as μmol $FeSO_4$/mg of dry peptide

[2] Expressed as μmol Trolox/mg of dry peptide

[3] Expressed as μmol Na_2EDTA/mg of dry peptide

The peptides represent approximately 70% of the total detected protein in common bean NDF.

Figure Captions

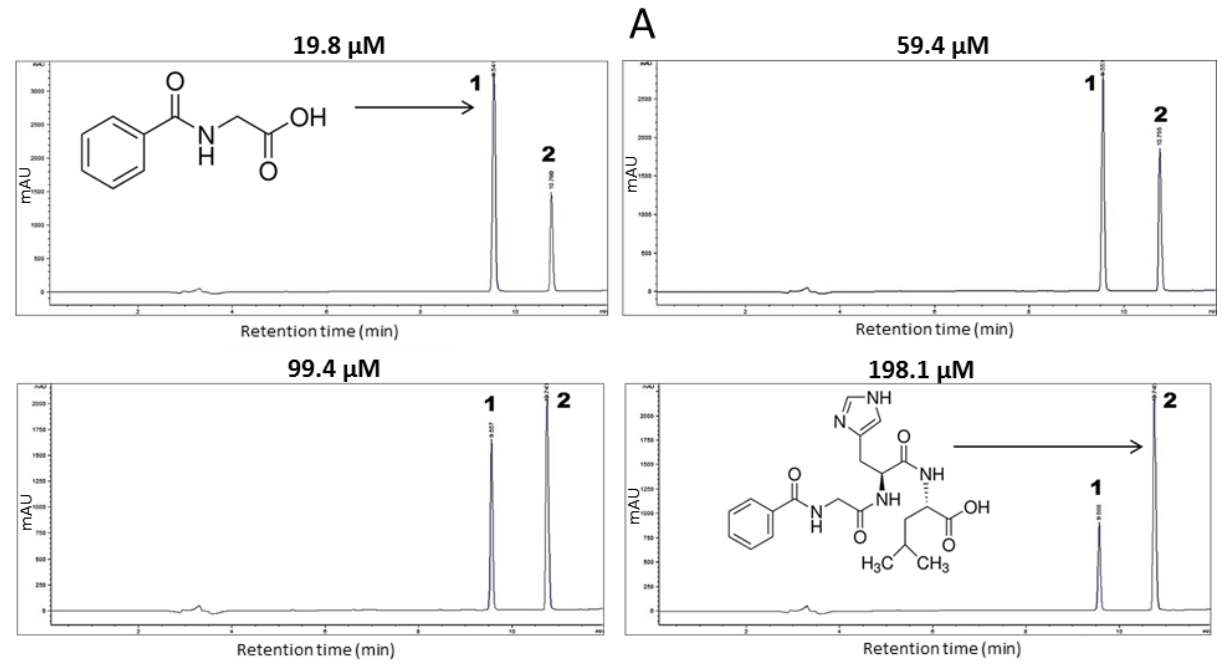
Figure 1 A) Representative chromatograms of the ACE inhibition by GLTSK peptide at different concentrations; where identified peak 1 shows hippuric acid (HA) and 2 for hippuryl-histidyl-leucine (HHL). B) ACE inhibition curves of the peptides. C) Summary for IC_{50} values of ACE inhibitory captopril and peptides present in common bean non-digestible fraction. The results are expressed as the mean ± standard deviation of at least two independent experiments. IC_{50}: Required concentration to inhibit enzymatic activity of ACE in 50%; the lower the value the more effective the peptide.

Figure 2 Effect of the pepsin/pancreatin digestion on peptides present in common bean non-digestible fraction regarding A) the ratio of ACE inhibition percentage/concentration of peptide and B) structure stability of peptides measured as peak area. The results are expressed as the mean ± standard deviation of at least two independent experiments. Different letters by pair of bars mean significant difference ($p<0.05$) as determined by Student's T-test.

Figure 3 Interaction between peptides present in beans to inhibit ACE A) synergistic interaction of GLTSK and MTEEY peptides and B) additive effects between GLTSK and MPACGSS, and C) additive effects between MTEEY and MPACGSS peptides. Bar plots represent the mean ± standard deviation of at least two independent experiments, with only the expected and combined values were compared statistically. ** mean significant difference ($p<0.05$). The lower the values the more potency.

Figure 4 Molecular docking diagrams exemplifying the analysis of GLTSK peptide showing A) best pose of the peptide (spheres) inside the ACE catalytic site, B) best pose of the peptide (sticks) with the interacting side chains of the catalytic site, C) distances of the peptide to the residues of the catalytic cavity which presented interaction, and D) diagram of the interaction type of the peptides with the amino acid residues of the catalytic site.

Figure 1



C

Compound	IC_{50} (µM)
Captopril	17.5 ± 0.04^{f}
GLTSK	65.4 ± 0.38^{e}
MTEEY	90.3 ± 0.35^{d}
LSGNK	130.6 ± 1.56^{c}
GEGSGA	113.7 ± 2.97^{b}
MPACGSS	191.5 ± 1.65^{a}

Figure 2.

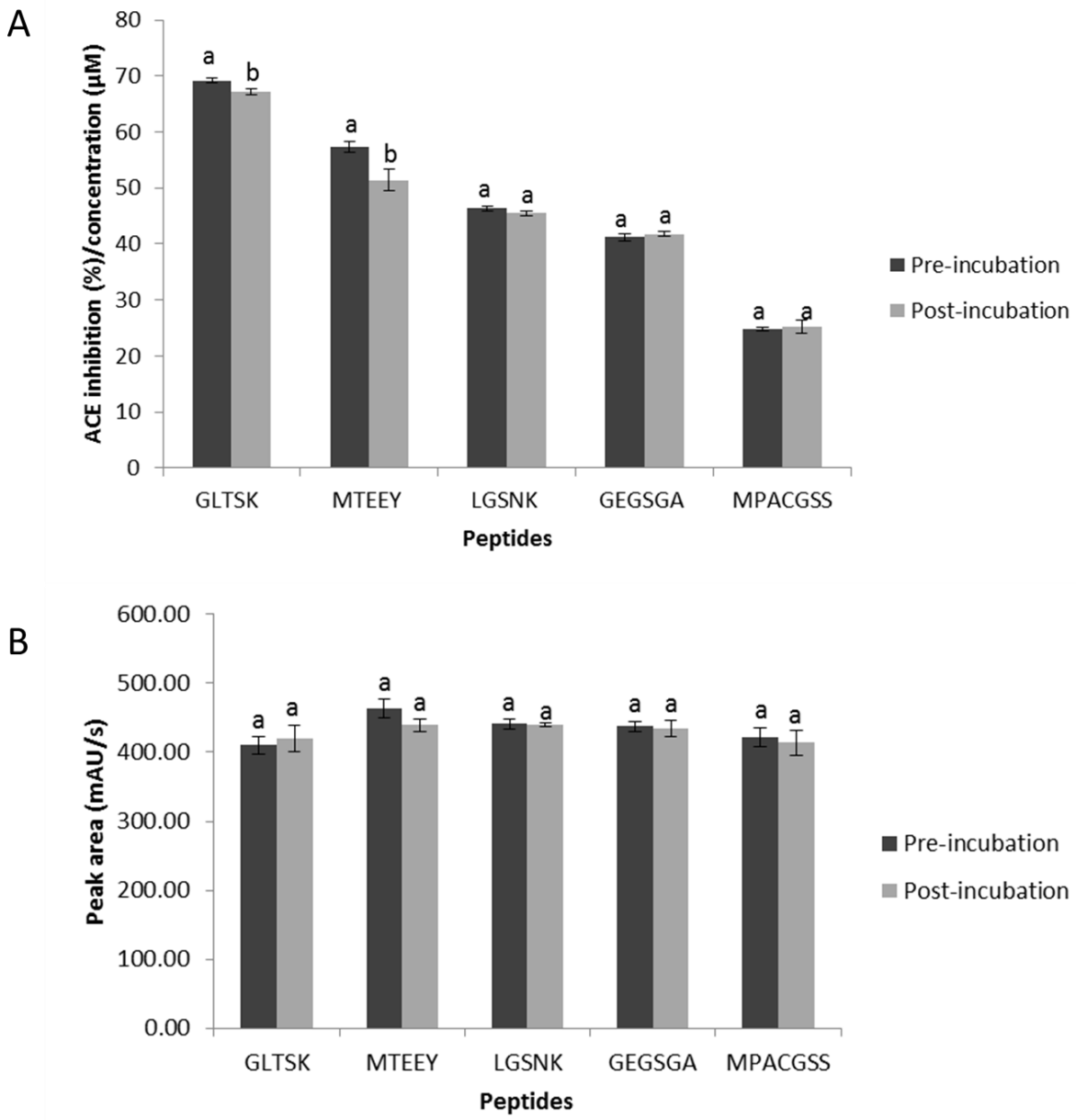


Figure 3.

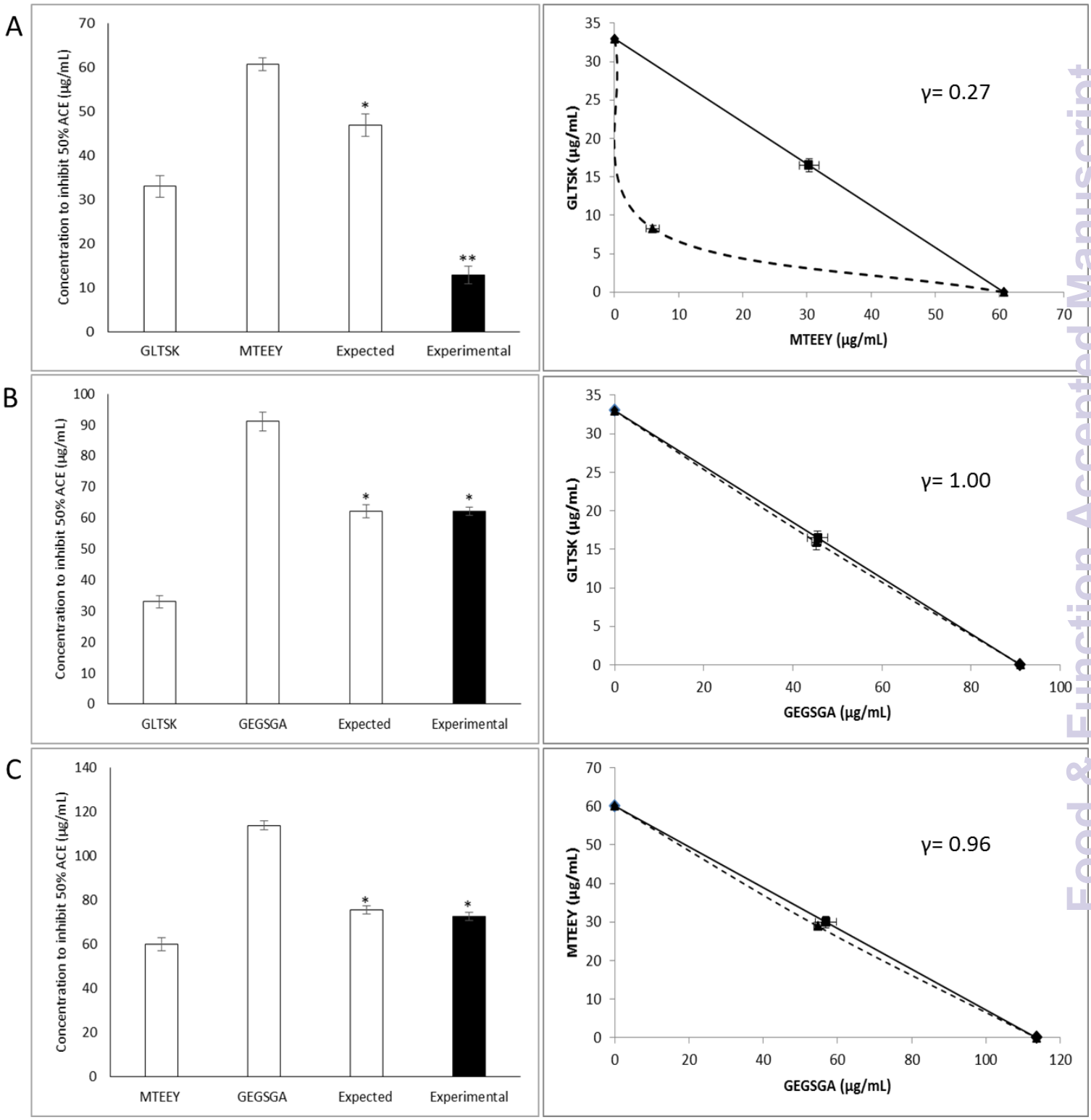


Figure 4